Int. J. Cancer: **85**, 391–397 (2000)

Publication of the International Union Against Cancer

NOVEL BREAST-TUMOR-ASSOCIATED MUC1-DERIVED PEPTIDES: CHARACTERIZATION IN $D^{b-/-}$ × β2 MICROGLOBULIN (β2m) NULL MICE TRANSGENIC FOR A CHIMERIC HLA-A2.1/D^b-β2 MICROGLOBULIN SINGLE CHAIN

Lior CARMON[1], Khaled M. EL-SHAMI[1], Adrian PAZ[1], Steve PASCOLO[3], Esther TZEHOVAL[1], Boaz TIROSH[1], Romelin KOREN[4], Michael FELDMAN[1], Mati FRIDKIN[2], François A. LEMONNIER[3] and Lea EISENBACH[1]*

[1]*Department of Immunology, Weizmann Institute of Science, Rehovot, Israel*
[2]*Department of Organic Chemistry, Weizmann Institute of Science, Rehovot, Israel*
[3]*AIDS-Retrovirus Department, Antiviral Cellular Immunity Unit, Pasteur Institute, Paris, France*
[4]*Department of Pathology, Rabin Medical Canter, Golda Campus, Rabin Medical Center of Israel, Petah Tiqva, Israel*

The MUC1 protein was found to be up-regulated in a spectrum of malignant tumors. T-cell responses to the MUC1 extracellular tandem repeat array (TRA) were observed in murine models as well as in breast-carcinoma patients. In the present study, we evaluated the anti-tumor potential of HLA-A2.1-motif-selected peptides from non-TRA domains of the molecule. Peptide immunogenicity was examined in the $D^{b-/-}$ × β2 microglobulin (β2m) null mice transgenic for a modified HLA-A2.1/D^b-β2 microglobulin single chain (HHD mice). Our results show the existence of 3 novel HLA-A2.1-restricted MUC1-derived cytotoxic T-lymphocyte (CTL) epitopes. These peptides are processed and presented by the HHD-transfected breast-tumor cell line MDA-MB-157. Moreover, CTL induced by these 3 peptides show higher lysis of target cells pulsed with breast-carcinoma-derived peptides than of targets pulsed with normal breast-tissue-derived peptides. These data suggest an important role for non-TRA MUC1-derived peptides as inducers of a MHC-restricted CTL reaction to a breast-carcinoma cell line and patient-derived tumor extracts. *Int. J. Cancer* 85:391–397, 2000.

The treatment or prevention of cancer with vaccines has been an objective vigorously sought since the first vaccines against infectious diseases were developed. This quest is based on 3 premises: first, there exist qualitative and quantitative differences between tumor cells and most normal cells; second, the immune system is ideally suited for identifying these differences; finally, the immune system could be instructed to recognize these differences and to effectuate tumor rejection. Cytotoxic T lymphocytes (CTL) directed against tumor-associated peptides presented by MHC-class-I molecules constitute powerful effectors of the immune system against tumors. These peptides are usually 8 to 10 amino acids long, with 2 to 3 primary anchor residues that interact with the MHC-class-I molecules and 2 to 3 amino-acid residues that engage the T-cell receptor (Rammensee *et al.*, 1993). CTL lines are among the tools used for the identification and characterization of TAA epitopes. However, there are several disadvantages in utilizing this strategy. First, it is difficult to establish carcinoma-associated CTL lines from patients' peripheral-blood lymphocytes (PBL). Second, CTL lines derived from cancer patients may represent, at least partially, the repertoire of the anergized immune system. Finally, the *in vitro* propagation of CTL lines might enhance sporadic clones surviving culture conditions rather than specific anti-tumor clones. An alternative strategy, which bypasses these potential pitfalls, is based on the use of HLA transgenic mice.

A number of studies have compared the CTL repertoire of defined peptides, restricted by HLA-A2.1 in human PBL from HLA-A2.1-matched patients, with CTL induced in HLA-A2.1-transgenic mice. Good concordance and an overlapping repertoire were found between the endogenous HLA-A2.1 and the murine transgenic HLA-A2.1 CTL repertoire, confirming the potential utility of such transgenic mice in the identification of human CTL epitopes (Shirai *et al.*, 1995; Wentworth *et al.*, 1996). Although vaccination with defined peptides in HLA transgenic mice showed a repertoire overlapping that of human PBL, vaccination of HLA-transgenic mice with multi-epitope proteins induced dominant murine H-2-restricted responses (Barra *et al.*, 1993). In order to obtain complete HLA-restricted responses, we used the $D^{b-/-}$ × β2 microglobulin (β2m) null mice transgenic for a recombinant HLA-A2.1/D^b-β2 microglobulin single chain (HHD mice) (Pascolo *et al.*, 1997). These mice combine classical HLA transgenesis with selective destruction of murine H-2. Hence, unlike the classical HLA transgenics, these mice showed only HLA-A2.1-restricted responses with multi-epitope proteins such as intact viruses. In addition, HHD mice selected the same immunodominant CTL epitopes as recognized by PBL in influenza-infected HLA-A2.1 individuals (Pascolo *et al.*, 1997). Hence, these mice are presumably a useful tool for the identification and characterization of potential tumor-derived HLA-A2.1-restricted CTL epitopes, as a step toward anti-tumor-antigen-based vaccine preparation.

Several methods have been employed to identify tumor-associated CTL epitopes. One such method is the identification of CTL epitopes subsequent to the search for MHC-binding motifs in known putative TAAs, as in the case of the breast-carcinoma-associated HER2/neu receptor (Fisk *et al.*, 1995) or the colorectal-tumor-associated carcino-embryonic antigen (CEA) (Ras *et al.*, 1997). In this regard, a potential breast-cancer-associated target is the MUC1 antigen. This polymorphic epithelial mucin, encoded by the MUC1 gene, is a high-molecular-weight transmembranal glycoprotein over-expressed in a broad range of tumors (Graham *et al.*, 1996; Ho *et al.*, 1993). It was found that the growth rate of primary breast tumors induced by the polyoma middle-T antigen is significantly slower in MUC1 null mice, suggesting that MUC1 might play a role in the progression of mammary carcinoma (Spicer *et al.*, 1995). A major feature of the MUC1 molecule is the presence of a highly immunogenic extracellular tandem repeat array (TRA) heavily O-glycosylated at serine and threonine residues. Altered carbohydrate structure of MUC1 in breast-cancer cells is probably responsible for the exposure of core epitopes within MUC1, specifically recognized by monoclonal antibodies (MAbs), by non-MHC-restricted CTL (Barnd *et al.*, 1989), HLA-A11-restricted (Domenech *et al.*, 1995) and HLA-A2.1-restricted CTL responses to the extracellular TRA (Apostolopoulos *et al.*, 1997). However, it was suggested that mucins can either inhibit (Van de Wiel-van Kemenade *et al.*, 1993) or actively suppress cell-mediated responses against glycosylated TRA (Fung and Longenecker, 1991). Agrawal *et al.* (1998) have shown that synthetic

Grant sponsors: Israeli Ministry of Science; Israeli Ministry of Health; Israeli Cancer Association.

*Correspondence to: Department of Immunology, Weizmann Institute of Science, Rehovot 76100, Israel. Fax: +972-8-9344141. E-mail: lceisen@weizmann.weizmann.ac.il

Received 4 January 1999; Revised 19 March 1999

peptides derived from MUC1 TRA cause suppression of human T-cell proliferative responses. These observations point to an ambiguous role of MUC1 TRA in T-cell activation. In the present study, we describe the characterization of 3 novel HLA-A2.1-restricted MUC1-derived CTL epitopes. These peptides are not deduced from the extracellular TRA and are processed and presented by a breast-tumor cell line. Moreover, CTL induced against these peptides lysed target cells pulsed with breast-carcinoma-derived peptide extracts more efficiently than target cells pulsed with normal-breast-derived peptides. These results suggest an important role for non-TRA-MUC1-derived peptides as inducers of HLA-restricted CTL responses to a breast-carcinoma cell line as well as tumor extracts.

MATERIAL AND METHODS

Mice

The derivation of HLA-A2.1/D^b-β2 monochain, transgenic, H-2D^b × β2m double-knockout mice (named HHD mice) has been described by Pascolo *et al.* (1997).

Tumor cells

MDA-MB-157 is a human breast-cancer cell line negative for expression of HLA-class-I molecules (Young *et al.,* 1974). The MDA-MB-157-HHD clone is a HHD transfectant of MDA-MB-157 cells. RMA-S is a TAP-2-deficient lymphoma clone of C57Bl/6 origin. The RMA-S-HHD clone is a HHD transfectant of RMA-S cells. The RMA-S-HHD-B7.1 clone is a HHD transfectant expressing the murine B7.1 co-stimulatory molecule. T2 is a TAP-2-deficient lymphoblastoid line of HLA-A2.1 genotype.

MDA-MB-157 cells were maintained in DMEM containing 10% FCS, 1 mM glutamine, combined antibiotics, 1 mM sodium pyruvate and 1% non-essential amino acids. MDA-MB-157-HHD transfectants were maintained in the same medium supplemented with 500 μg/ml of geneticin (Life Technologies, Paisley, UK). RMA-S and T2 cells were maintained in RPMI 1640 containing 10% FCS and combined antibiotics.

Peptide synthesis

Peptides were synthesized on an ABIMED AMS 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany), employing the α-N-fluorenylmethoxy-cartonyl (Fmoc) strategy following the commercially available manufacturer's protocols. Peptide-chain assembly was conducted on a 2-chlorotrityl chloride resin (Novabiochem, Laufelfingen, Switzerland). Crude peptides were purified to homogeneity by reversed-phase HPLC on a semi-preparative silica C-8 column (250 × 10 mm; Lichnonorb RP-8; Merck, Darmstadt, Germany). Elution was accomplished by a linear gradient established between 0.1% TFA in water and 0.1% TFA in 70% acetonitrile in water (v/v). Composition of the products was determined by amino-acid analysis (automatic amino-acid analyzer; Dionex, Sunnyvale, CA) after extraction acid hydrolysis. Molecular weight was ascertained by mass spectrometry (VG Tofspec; Laser Desorption Mass Spectrometry; Fisons, Manchester, UK).

Preparation of tumor-extract peptides

Total acid-extracted peptides of breast tumor or of normal adjacent tissues were prepared from a pool of 5 to 6 post-surgical breast-cancer specimens. Non-necrotic (1–2 cm) tumor masses were homogenized in PBS, 0.5% Nonidet P-40, 10 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 8 μg/ml aprotonin and 0.5 mM PMSF, and homogenized using a glass-Teflon homogenizer. Following further stirring for 30 min at 4°C, the homogenates were titrated with 10% TFA to a final concentration of 0.1% TFA and stirred for another 30 min at 4°C. After ultracentrifugation for 30 min at 130,000g the supernatants were applied to Sephadex G25 columns and fractions were monitored at optical density (OD) 230 nm. Peptide fractions below 10 kDa were pooled, lyophilized and further fractionated by Centriprep 3 centrifugation (Amicon, Beverly, MA). Lyophilized samples were dissolved in sterile double-distilled water, freed from TFA by repeated lyophilization and relative concentrations were monitored by determination of OD of 230 nm. The yields from both tumor and normal tissue were 130 to 160 OD of 230 nm/g. Following lyophilization, the peptide pool was dissolved in opti-MEM (Life Technologies) at 30 to 50 OD 230 nm/ml for further use.

Peptide loading for FACS analysis

Peptide loading of RMA-S-HHD transfectants was performed as follows. After the cells were washed 3 times in PBS, the surface expression of HHD monochain was stabilized by a 4-hr culture at 26°C. Synthetic peptides, or peptide extracts were added to 5 × 10^5 cells in 50 μl of opti-MEM (Life Technologies) to a concentration of 1 to 100 μM and/or 0.25 to 1 OD of 230 nm respectively. The cells were incubated for 2 to 3 hr at 37°C prior to FACS analysis (Becton Dickinson, Canberra, Australia).

Measurement of peptide binding by stabilization of cell-surface MHC and expression of MUC1

Peptide binding to HHD single chain was measured by stabilization of HHD on RMA-S-HHD transfectants, using an indirect FACS assay as follows: 5 × 10^5 peptide-loaded TAP2-deficient RMA-S-HHD cells (see peptide loading) were incubated with anti-HLA MAb for 30 min at 4°C. After the cells were washed with PBS-0.5% BSA + 0.1% sodium azide, the second Ab, goat anti-mouse-FITC (Jackson Laboratories, Bar Harbor, ME), was applied for 30 min at 4°C. Following washing, the amount of bound antibodies was detected by FACScan. The cell-surface expression of MUC1 on MDA-MB-157 was detected by the same FACS protocol using anti-MUC1 MAb H23 (a kind gift of Dr. Y. Keydar, Department of Cell Research and Immunology, Tel-Aviv University).

Mouse MAbs B-9-12, W6/32 (anti-HLA A,B,C) 28-14-8 (anti-H-2D^b α3 domain) and BB7.2 (anti-HLA-A2.1) were used for analysis.

Vaccination

Mice were immunized i.p. 3 times at 7-day intervals with 2 × 10^6 irradiated (5000 rad) tumor cells, or with irradiated peptide-loaded RMA-S-HHD-B7.1 transfectants. Peptide loading of RMA-S-HHD-B7.1 cells was performed as follows. The cells were washed 3 times in PBS, then cell-surface expression of HHD monochain was stabilized by 4-hr culture at 26°C. Synthetic peptides or peptide extracts were added to 10 × 10^6 cells in 1 ml of opti-MEM (Life Technologies) to a concentration of 100 μM or 1 OD of 230 nm respectively. The cells were incubated overnight at 26°C and for 3 additional hr at 37°C. Peptide-loaded RMA-S-HHD-B7.1 cells were irradiated (5000 rad), washed, re-suspended in PBS and injected into mice. In mixed synthetic vaccines, RMA-S-HHD-B7.1 cells were loaded separately with each peptide and pooled before vaccination.

In vitro *cytotoxicity assays*

Mice were immunized i.p. 3 times at 7-day intervals, with 2 × 10^6 irradiated (5000 rad) tumor cells or with peptide-loaded RMA-S-HHD-B7.1 transfectants. Spleens were removed on day 10 after the last immunization, and splenocytes were re-stimulated *in vitro,* either with irradiated tumor cells (for mice immunized with tumor cells) or with one third of the lymphocytes pulsed with 100 μM synthetic peptides or 1 OD 230-nm patient-derived extract in opti-MEM (Life Technologies) for 2 hr at 37°C, 5% CO_2. Re-stimulated lymphocytes were maintained in RPMI-HEPES medium containing 10% FCS, 1 mM glutamine, combined antibiotics, 1 mM sodium pyruvate, 10 mM HEPES, pH 7.4, 5 × 10^{-5} M β-mercaptoethanol and 1% non-essential amino acids for 5 days. Viable lymphocytes (effector cells) were separated by Lympholyte-M (Cedarlane, Hornby, Canada) centrifugation, re-suspended in RPMI-HEPES and admixed at different ratios with 5 × 10^3 ^{35}S-methionine-labeled peptide-loaded RMA-S-HHD cells. CTL assays were performed in U-shaped microtiter wells, at 37°C, 5%

CO_2 for 5 hr. Cultures were terminated by centrifugation at 250g for 10 min at 4°C. A total of 100 µl of the supernatants was mixed with scintillation fluid and measured in a beta counter (Becton Dickinson). Percentage of specific lysis was calculated as follows: % lysis = (cpm in experimental well − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release) ×100. Spontaneous release was determined by incubation of 100 µl labeled target cells with 100 µl of medium. Maximal release was determined by lysis of target cells in 100 µl 0.1 M NaOH.

RESULTS

Screening for MUC1-derived HLA-A2.1-restricted peptides

In this study we focused on the identification of HLA-A2.1 TAA peptides derived from the entire MUC1 molecule. The MUC1 amino-acid sequence was screened for potential HLA-A2.1-binding peptides utilizing the program for "independent binding of individual peptide side-chains" (Parker *et al.*, 1994). Eight MUC1-derived peptides were selected and synthesized. Table I summarizes MUC1-derived peptide positions and their calculated binding scores. These peptides, of 9 residues, are derived from the signal peptide, cytoplasmic and extracellular domains of the MUC1 protein. Yet none of these peptide sequences is located within the immunogenic TRA domain. Among the peptides, only the MUC1/B5 peptide has an identical sequence with the MUC1 murine homolog.

HLA-A2.1 binding of MUC1-derived peptides was measured by FACS analysis. The selected peptides were loaded on the murine TAP2-deficient RMA-S-HHD transfectants (HLA-A2.1/D^b-β2m single chain) and MHC stabilization was monitored (Fig. 1). Although all peptides bound efficiently in the 1-to-100 µM range, 3 peptides, MUC1/D6, MUC1/E6 and MUC1/A7, exhibited high binding affinity. We obtained similar binding affinities of these peptides when they were loaded on the human TAP2-deficient T2 cells expressing endogenous HLA-A2.1 molecules (data not shown). To monitor the relative levels of HLA-A2.1-binding peptides in tumor- and normal-breast-tissue-extracted peptides, RMA-S-HHD cells were loaded with equivalent amounts of extracts and HHD stabilization was determined in comparison with tyrosinase-stabilized HHD (Fig. 2). The data show that similar levels of HLA-A2.1 binding peptides exist in either tumor-derived or normal tissue-derived peptide extracts.

CTL response in HHD mice induced by pools of MUC1 peptides

Synthetic peptides corresponding to the MUC1 TRA epitopes were shown to induce CTL in patients (Domenech *et al.*, 1995) as well as in HLA-A2.1/K^b transgenic mice (Apostolopoulos *et al.*, 1997). We first examined the lysis patterns of each of the individual peptides loaded on target cells following immunization with a pool of all MUC1-derived synthetic peptides (Fig. 3*a*). CTL results showed significant lysis of RMAS-HHD target cells loaded either with the MUC1/D6 peptide (38%) or with the MUC1/A7 peptide (32%). Lysis of 15% was obtained with the MUC1/E6 and MUC1/F6 peptides, while other peptides showed only background lysis. Immunization of HHD mice with single MUC1 peptides showed 40% lysis for MUC1/A7 or MUC1/D6 and 75% lysis for MUC1/E6, while RMA-S-HHD targets loaded with the melanoma-specific HLA-A2.1-binding tyrosinase peptide showed background lysis (Fig. 3*b*). Hence, MUC1/D6, MUC1/A7 and MUC1/E6 conferred CTL susceptibility with possible immunogenic potential.

TABLE I – SELECTION OF MUC1-DERIVED HLA-A2.1-RESTRICTED PEPTIDES[1]

Peptide[2]	Position number[3]	Sequence[4]	Score[5]
MUC1/C6	31–40	LLLLTVLTV	1006.209
MUC1/D6	32–41	LLLTVLTVV	412.546
MUC1/F6	323–331	FLSFHISNL	226.014
MUC1/A5	442–451	LLVLVCVLV	118.238
MUC1/F4	441–450	ALLVLVCVL	74.536
MUC1/B5	519–528	SLSYTNPAV	69.552
MUC1/A7	412–421	NLTISDVSV	69.552
MUC1/E6	226–234	ALASTAPPV	69.552

[1]MUC1-derived peptides were selected according to the known consensus motifs for peptides bound by HLA-A2.1.–[2]Peptide designation.–[3]Position of first and last amino acid in the protein sequence.–[4]Amino-acid sequence of the peptides.–[5]Calculated score, estimating half the time for dissociation of the peptide-HLA complex.

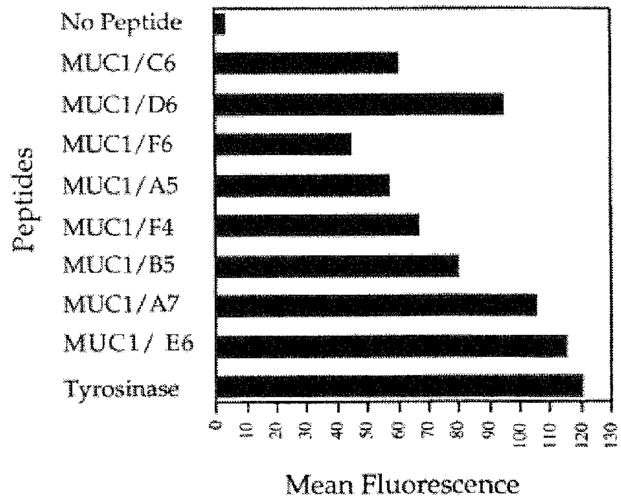

FIGURE 1 – Stabilization of cell-surface MHC by MUC1-derived peptides. MUC1-derived peptides were loaded at various concentrations (1–100 µM) on TAP2-deficient RMA-S-HHD cells as described, and indirect FACS analysis was performed by incubating 5 × 10^5 loaded cells with anti-HLA-A2.1 MAb BB7.2 for 30 min at 4°C. After the cells were washed with PBS-0.5% BSA + 0.1% sodium azide, the secondary Ab goat anti-mouse-FITC was applied for 30 min at 4°C. Following another wash, the amounts of bound antibodies were detected in a FACScan. Mean fluorescence at 100 µM peptide is shown. The HLA-A2.1-binding tyrosinase-derived peptide is presented as a positive control and unloaded RMA-S-HHD as a negative control. Results are representative of 3 similar experiments.

HLA-A2.1-restricted lysis of breast-carcinoma MDA-MB-157-HHD transfectants by CTL induced against patient-derived tumor extract or by anti-MUC1-derived-peptide-specific CTL

To determine the processing and presentation of MUC1-derived peptides by breast-carcinoma tumors, we selected the MDA-MB-157 cell line, which is characterized by high MUC1 expression and low HLA-class-I expression. FACS analysis of MDA-MB-157 and their HHD transfectants using H23 anti-MUC1 MAb showed high MUC1 expression (Fig. 4*a*). No class-I expression was detected on parental MDA-MB-157 cells stained with anti-HLA-A2.1 MAb, while HHD transfectants reacted with anti-HLA-A2.1 MAb and with anti-H-2D^b MAb directed against the α3 domain (Fig. 4*b,c*).

Both the parental tumor cell line and its HLA-A2.1-β2m single-chain transfectant (MDA-MB-157-HHD) were used as targets for CTL lysis (Fig. 5). Mice were immunized either with RMA-S-HHD-B7.1 cells loaded with MUC1-selected peptides MUC1/D6, MUC1/A7 and MUC1/E6, or with peptide extract derived from patients' tumors (TE). Preferential lysis of the MDA-MB-157-HHD cell line was compared with parental cells by anti-MUC1-activated lymphocytes or by tumor-extract-activated lymphocytes indicating both breast-associated as well as MHC-restricted lysis. These data strongly suggest that processing and presentation of non-TRA-associated MUC1-derived peptides take place in MDA-MB-157-HHD breast-carcinoma cells. Further analysis showed inhibition of lysis by anti-HLA MAb w6/32 (data not shown). Moreover, specific lysis of MDA-MB-157-HHD by CTL directed against fresh tumor-extracted peptides indicated an

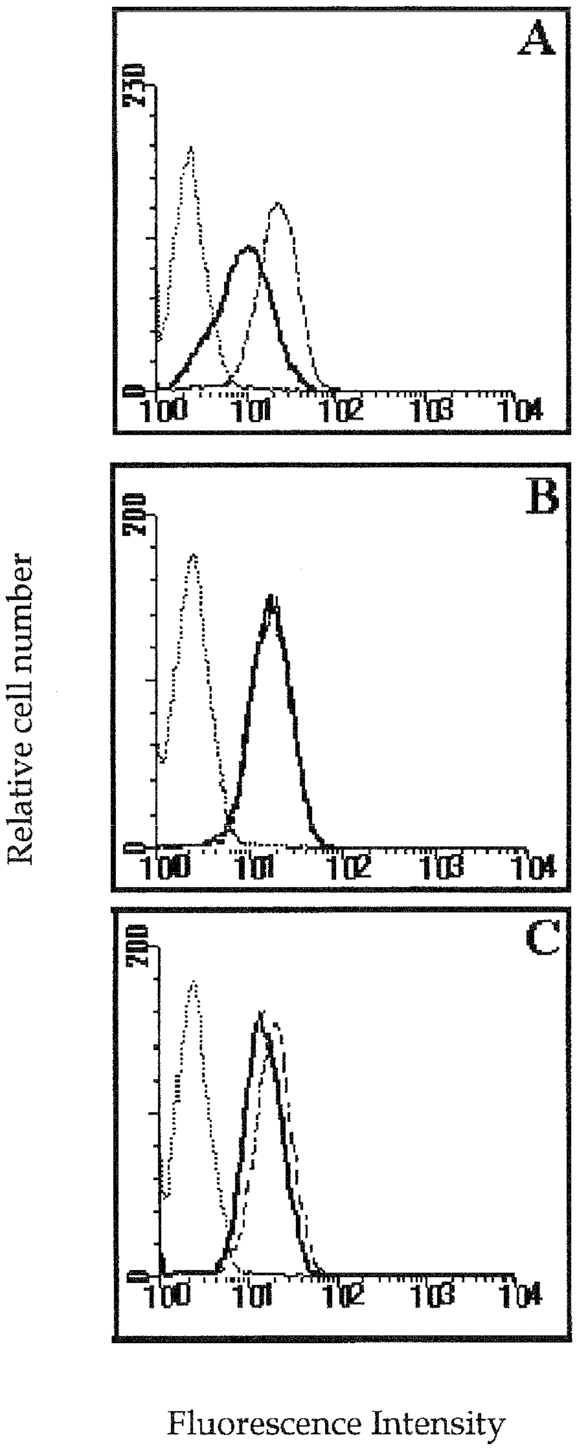

FIGURE 2 – Stabilization of cell-surface MHC by tumor- and normal-tissue-extracted peptides. Peptides >3 kDa were prepared as described. RMA-S-HHD cells were loaded as above, in the presence of 0.25 OD 230 nm (full line) or 1 OD 230 nm (broken line), stained with BB7.2 MAb followed by goat anti-mouse-FITC and monitored by FACScan. Background staining with the second Ab is shown as a dotted line. *(a)* Tyrosinase peptide; *(b)* normal tissue-derived peptides; *(c)* tumor-derived peptides. Results are representative of 2 similar experiments.

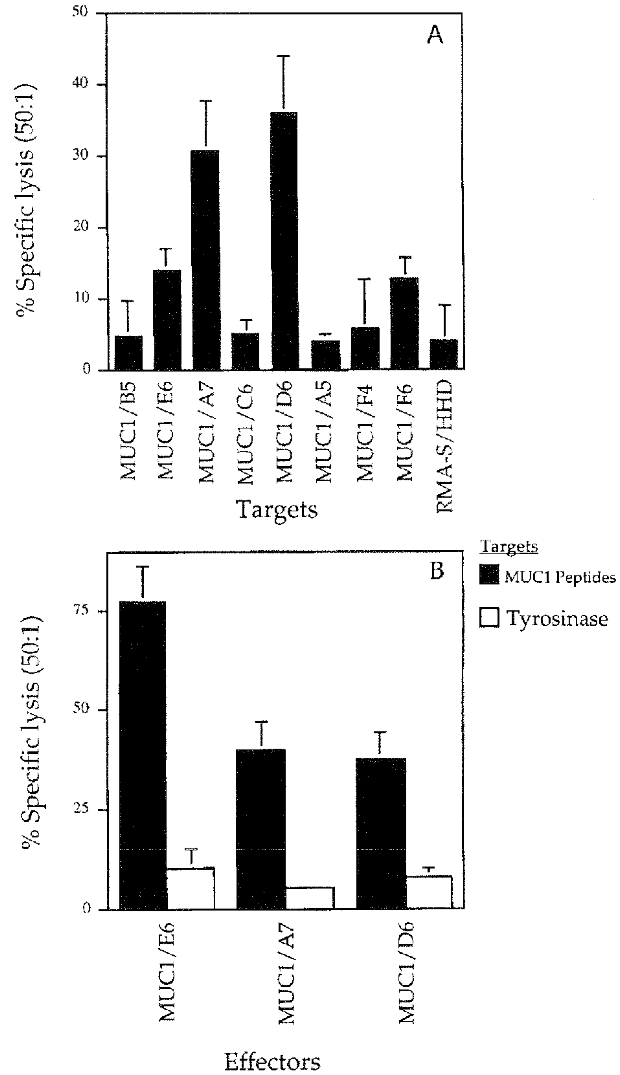

FIGURE 3 – Immunogenicity of MUC1-derived peptides in HHD mice. Mice were immunized i.p. 3 times at 7-day intervals, with 2×10^6 irradiated (5000 rad) peptide-loaded TAP2-deficient RMA-S-HHD-B7.1 cells. *(a)* The cells were loaded separately with individual peptides, washed and pooled before immunization. *(b)* The cells were loaded with single peptides and injected individually. Spleens were removed on day 10 and splenocytes were re-stimulated *in vitro* by 100 μM MUC1-derived peptides in opti-MEM for 2 hr at 37°C, 5% CO_2, followed by re-stimulation of lymphocytes for 4 more days in RPMI-HEPES as described. CTL assays were performed on day 5 with individual MUC1-derived peptides loaded on RMA-S-HHD as targets. Unloaded RMA-S-HHD or tyrosinase-loaded targets were used as negative controls. The effector-to-target ratio of 50:1 is shown. Specific lysis of all 3 peptides, E6, A7 and D6, is statistically significant ($p < 0.001$) compared with lysis of the tyrosinase peptide. Results represent the average of 3 similar experiments.

overlapping peptide repertoire between the breast-tumor cell line and breast-tumor explants.

CTL induced by total-tumor-extracted peptides lyse RMA-S-HHD pulsed with MUC1-derived peptides

To test whether MUC1/D6, MUC1/A7 and MUC1/E6 peptides are dominant epitopes in breast-carcinoma-patient-derived peptide extracts, CTL assay was performed utilizing CTL against breast-

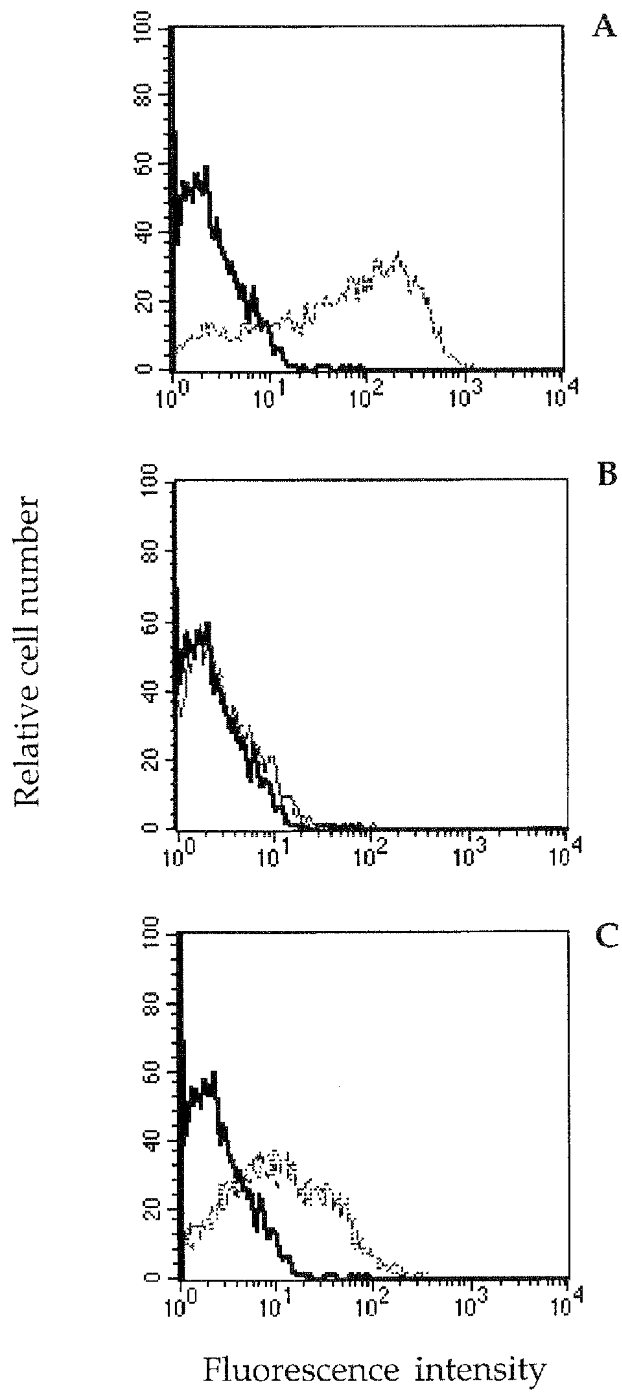

FIGURE 4 – Expression of MUC1 and HLA-A2.1 single chain on MDA-MB-157-HHD cells. MDA-MB-157-HHD transfectants were evaluated for expression of MUC1 antigen *(a)*. Wild-type MDA-MB-157 cells were evaluated for expression of HHD (HLA-A2.1 single chain) *(b)* compared with HHD expression by MDA-MB-157-HHD transfectants *(c)*. FACS staining for HHD and for MUC1 molecules was performed as described in Material and Methods. Anti-HLA-A2.1 MAb BB7.2 (dashed line) and anti-D^b-α3 domain MAb 28-14-8 (dotted line) were used for HLA-A2.1 single-chain expression. Anti-MUC1 MAb H23 was used for monitoring MUC1-antigen expression. Results are representative of 5 similar experiments.

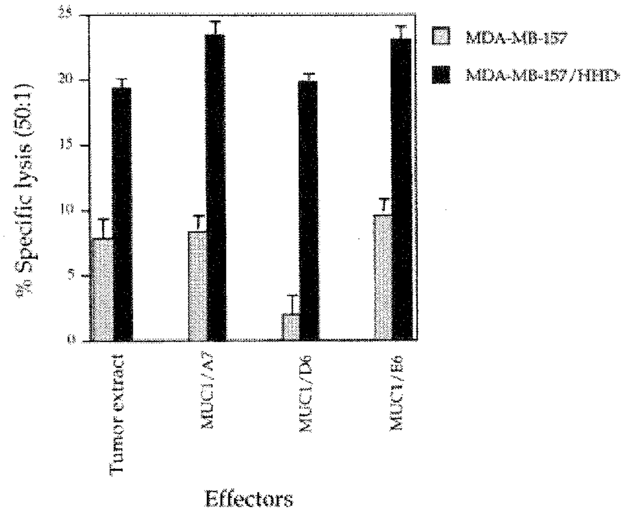

FIGURE 5 – HLA-A2.1-restricted lysis of the breast-carcinoma cell line MDA-MB-157-HHD transfectant by CTL against MUC1-derived peptides and patient-derived tumor extract. Mice were immunized as described in Material and Methods with patient-derived tumor extract or with MUC1/A7, MUC1/E6 and MUC1/D6 peptides loaded on RMA-S-HHD-B7.1 transfectants. Lysis of MDA-MB-157 cells and MDA-MB-157-HHD breast-carcinoma transfectants was monitored by CTL assays. Specific lysis of HHD-transfected MDA-157 was significantly higher ($p < 0.0006$) than that of wild-type MDA-157 target cells. E:T 50:1. Results are representative of 3 similar experiments.

tumor-peptide extracts as effectors against target cells presenting MUC1 peptides (Fig. 6). As a positive indicator, we used target cells pulsed with an HLA-A2.1-binding HER2/neu-derived peptide KIFGSLAFL, shown to be an immunodominant epitope, recognized by ovarian-specific and breast-specific CTL lines (Fisk *et al.*, 1995). Target cells pulsed with the melanoma/melanocyte-specific peptide of tyrosinase served as a negative control. All 3 MUC1-derived peptides and HER2/neu-derived peptide, but not the HLA-A2.1 melanoma-associated peptide tyrosinase, could be recognized and lysed by anti-tumor extract CTL. RMA-S-HHD cells loaded with normal breast extract showed 40 to 60% of the lysis induced against tumor-extract-loaded targets in different experiments (Fig. 6). This result is not surprising, since a major portion of the tumor-extracted-peptide repertoire consisted of normal-breast-tissue peptides. Yet, it indicates that some unique or over-expressed peptides in the tumor extract are immunogenic in HHD mice.

CTL induced by MUC1-derived peptides lyse RMA-S-HHD pulsed with tumor extract more efficiently than those pulsed with normal-tissue extract

A crucial parameter for selection of TAA-peptide-based vaccines is their expression levels in tumors as compared with those in normal tissues. Since the MUC1 protein is known to be over-expressed in tumors with no tumor-specific mutation, it is relevant to examine the abundance of the MUC1 peptides in patient-derived normal-breast-tissue extract (NE) in comparison with that in tumor extract (TE). CTL generated against MUC1 peptides MUC1/D6, MUC1/A7 and MUC1/E6 showed 2.7- to 7-fold higher reactivity to TE *vs.* NE (Fig. 7). Preferential lysis of tumor-peptide-loaded *vs.* normal-tissue-peptide-loaded targets could be observed upon vaccination with breast-tumor-extract peptides, indicating a degree of specificity between normal and tumor tissues. These results suggest that MUC1/D6, MUC1/A7 and MUC1/E6 are tumor-associated antigen peptides and that CTL induced against these peptides might preferentially lyse tumors while sparing normal tissues.

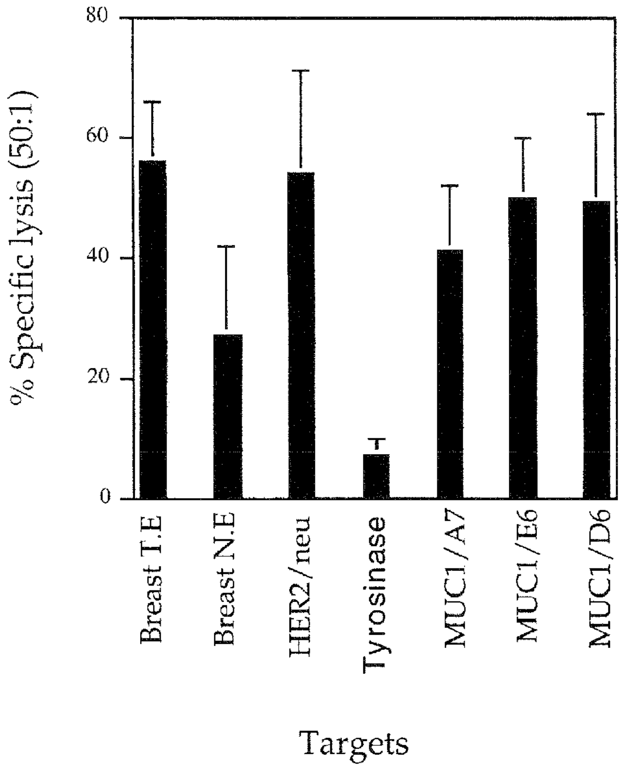

FIGURE 6 – Peptide-specific CTL responses in patient-derived-tumor-extract-immunized mice. CTL assays utilizing anti-patient-derived-tumor-extract-activated lymphocytes were performed as described in Material and Methods. MUC-1, HER2/neu- or tyrosinase-derived synthetic peptides as well as tumor- and normal-tissue-derived peptide extracts loaded on RMA-S-HHD served as targets. CTL lysis of tumor extract, as well as of MUC1-peptide-loaded targets, was significantly higher ($p < 0.003$) than lysis of tyrosinase-loaded targets, as was lysis of normal breast-tissue extract ($p < 0.04$). T.E, tumor extract; N.E, normal extract; E:T, 50:1. Results represent the average of 3 similar experiments.

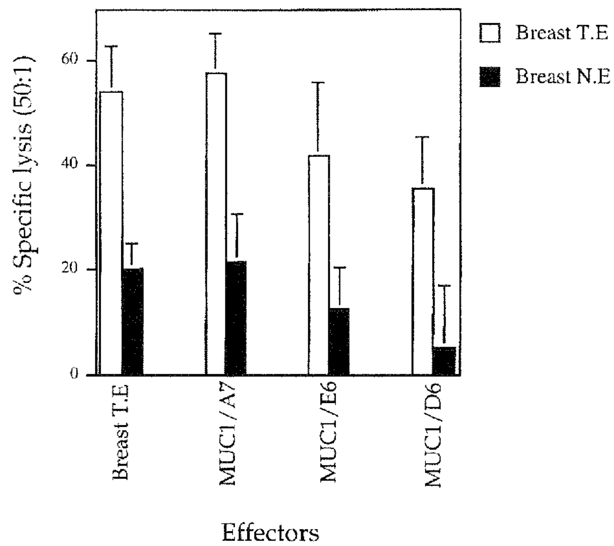

FIGURE 7 – Differential lysis of targets loaded with tumor- or normal-breast-tissue-derived peptides by anti-MUC1-induced CTL. CTL assays utilizing anti-MUC1/A7, -MUC1/E6 and -MUC1/D6 or anti-tumor-extract-peptide-activated lymphocytes were performed as described in Material and Methods, using patient-derived-tumor-extract-loaded and normal-tissue-extract-loaded RMA-S-HHD as targets. Specific lysis on tumor extract by CTLs induced by vaccination with different peptides was significantly higher than lysis of normal extract ($p < 0.01$). T.E, tumor extract; N.E, normal extract; E:T, 50:1. Results represent the average of 3 similar experiments.

DISCUSSION

The MUC1 tumor-associated antigen has been a focus of interest as an antigen in tumor immunology and immunotherapy, as well as for its possible role in tumorigenesis, metastasis and in signal transduction (Graham *et al.*, 1996; Ho *et al.*, 1993). In the current study, we examined the potential anti-tumoral use of novel MUC1-derived peptides. To this end, we used HHD mice, which not only express the human HLA-A2.1 class-I allele but also lack the ability to present murine epitopes, by virtue of a double knock-out deletion of the murine β2 microglobulin and D^b genes. Since the whole class-I-restricted-T-cell repertoire is selected on HLA-A2.1, these mice constitute a suitable animal model for the investigation and characterization of human tumor antigens through induction of HLA-restricted CTL responses. Eight HLA-A2.1-restricted peptides derived from the MUC1-protein sequence were selected according to their calculated MHC-binding affinities (Table I, Fig. 1). This approach has been used in a number of studies. Sette *et al.* (1994) showed a positive correlation between binding affinity and immunogenicity of potential CTL epitopes, while Vitiello *et al.* (1997) showed similar T-cell-epitope selection following immunization with HBV-motif-predicted class-I peptides or by HBV DNA immunization. Our data support these findings: MUC1/A7, MUC1/D6 and MUC1/E6 peptides show high MHC-binding affinity, positively correlated with preferential immunogenic properties in CTL assays. These results confirm the central role of MHC-binding affinity in determining immunodominance. Anti-MUC1/D6, -MUC1/A7 and -MUC1/E6-activated lymphocytes preferentially lysed the breast-carcinoma cell line MDA-MB-157-HHD (Fig. 5). This rules out the possibility of false-positive lysis due to exogenous pulsing of target cells, and strongly supports the possibility that MUC1/D6, MUC1/A7 and MUC1/E6 peptides are processed and presented in the MDA-MB-157-HHD breast-carcinoma cell line. Moreover, CTL induced against peptide extracts of fresh human tumors show HLA-A2.1-restricted lysis of the MDA-MB-157-HHD cell line (Fig. 5). Since the identity of the cross-reactive peptides in tumor-extract peptides and the cell line are unknown, testing of CTL induction by immunization with tumor-extract peptides against targets pulsed with the 3 MUC1 peptides confirmed that these CTL contained sub-populations that recognized MUC1 peptides, as well as CTL that identified a HER2/neu peptide (Fig. 6). Moreover, CTL induced against tumor-extract peptides lyse target cells pulsed with tumor-derived peptides more efficiently than target cells pulsed with normal-tissue-derived peptides (Figs. 6, 7), though equivalent concentrations of both extracts contain similar levels of HLA-A2.1-binding peptides (Fig. 2). The largest group of potential TAA peptides are those derived from proteins expressed in normal tissues as well as in tumors. Hence, a crucial parameter for selection of TAA-peptide vaccines is their frequency in tumors as compared with that in normal tissues. Our present data showed 2.7- to 7-fold higher CTL reactivity to tumor peptides *vs.* normal-tissue peptides generated against MUC1-selected peptides MUC1/D6, MUC1/A7 and MUC1/E6 (Fig. 7). Preferential recognition of tumor peptides but not normal peptides was also found upon vaccination with breast-tumor-extract peptides. These results reflect observations showing different gene-expression patterns in tumors *vs.* normal tissues (Zhang *et al.*, 1997); moreover, they emphasize the possibility of inducing anti-tumor immunity in normal tumor-associated over-expressed proteins.

Many studies have aimed to improve the anti-tumoral potential of MUC1 immunogenic TRA. Indeed, restricted or non-restricted CTL reactivity to TRA epitopes has been reported (Barnd *et al.*, 1989; Domenech *et al.*, 1995; Apostolopoulos *et al.*, 1997). However, TRA might suppress T cells, and its role in T-cell activation is controversial (Fung and Longenecker, 1991; Agrawal *et al.*, 1998). Moreover, an isoform of MUC1 lacking the tandem repeat array was found to be preferentially expressed on breast-cancer tissues (Baruch *et al.*, 1997). We reasoned that MUC1-antigen epitopes derived from outside the TRA may be advantageous, since this part of the molecule appears to play an important role in the transformation process and is therefore less likely to be selected out in the context of antigen- or epitope-loss variants resulting from specific immune responses. Since an immune response to TRA is an early event, we speculate that high-affinity T-cell clones (probably MHC-unrestricted) recognize epitopes on the highly immunogenic TRA and are subsequently partially suppressed or undergo apoptosis. A second wave of high-affinity restricted T-cell clones and moderate-affinity unrestricted clones to TRA epitopes or to alternative MUC1 epitopes are activated and induce anti-tumor immunity. Such epitopes can be derived from full-length MUC1 cDNA or from other MUC1 alternatively spliced variants, and might be crucial for elimination of residual disease. In conclusion, we suggest a role for non-TRA-related MUC1/D6, MUC1/A7 and MUC1/E6 as tumor-associated-antigen peptides. It would be of interest to test the ability of these peptides to prime PBL in breast-carcinoma patients, as a step toward the establishment of MUC1-derived-peptide vaccines.

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